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Steatosis and Intrahepatic Hepatitis C Virus in Chronic Hepatitis

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Hepatic steatosis has been reported as one of the characteristics which discriminates hepatitis C from other forms of hepatitis, besides lymphoid follicles and bile duct damage. However, it is unclear whether or not the presence of hepatitis C virus (HCV) itself is associated with the development of steatosis. The possibility that the HCV itself is directly related to the development of steatosis was examined. The intrahepatic core protein levels, as a marker of the HCV load, were correlated with the presence of steatosis in 43 patients with chronic hepatitis C. Among 43 patients studied by Western blotting, the core protein was detected in the liver in 27 (62.8%). On the other hand, hepatic steatosis was observed in 21 (48.8%) of the 43 patients. Importantly, the core protein was detectable in 19 (90.4%) of the 21 patients with steatosis, while it was detected in only 8 (36.4%) of the 22 patients without steatosis ($P = 0.008$). However, serum HCV-RNA levels as determined by the Amplicor monitor were not significantly different between patients with and without steatosis. Multivariate analysis showed that the serum alanine aminotransferase level ($P = 0.013$), body mass index ($P = 0.038$), and intrahepatic HCV core protein positivity ($P = 0.038$) were the independent parameters best predictive of steatosis. These results indicate a close relationship between intrahepatic HCV and the development of steatosis, and suggest a possible role of the HCV itself or core protein in the pathogenesis of steatosis in human chronic hepatitis C. ***J. Med. Virol. 59:141–145, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: HCV; core protein; HCV-RNA; fatty change; Western blotting

INTRODUCTION

Hepatitis C virus (HCV) is the major etiologic agent of non-A, non-B hepatitis and frequently causes persistent infection, which is associated with chronic hepatitis, cirrhosis, and the development of hepatocellular carcinoma [Choo et al., 1989; Saito et al., 1990]. Chronic hepatitis C is characterized by several histologic features of the liver, which distinguish it from other forms of hepatitis: bile duct damage, lymphoid follicles, and steatosis [Scheuer et al., 1992; Bach et al., 1992; Lefkowitch et al., 1993; Czaja and Carpenter, 1993; Delladetsima et al., 1996]. In a comparative study, steatosis was observed in 72% of 50 patients with chronic hepatitis C compared to 19% of patients with autoimmune chronic active hepatitis [Bach et al., 1992]. In another study, large-droplet fatty change was observed at a significantly higher rate in patients with chronic hepatitis C than in those with chronic hepatitis B [Lefkowitch et al., 1993]. Little is known, however, about the role of HCV or its viral proteins in the pathogenesis of steatosis. Recently, several factors have been evaluated in relation to the presence of steatosis in chronic hepatitis C patients [Czaja et al., 1998]. It has been suggested that steatosis in chronic hepatitis C is related to the presence of the virus itself; however, the HCV load in the serum or liver of patients of hepatitis C with steatosis has not previously been investigated.

In the present study, host- and disease-associated factors including the HCV load in the liver and serum in patients with hepatitis C were evaluated and correlated these factors with the presence of steatosis. As an index of HCV load in the liver, the HCV core protein,

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TABLE I. Clinical and Biochemical Profile of 43 Patients*

	Hepatic steatosis		
	(–)	(+)	*P*-value
Number of patients	22	21	
Age (years)[a]	51–80 (67 ± 7.1)	54–74 (64 ± 5.1)	NS
Sex (male:female)	18:4	15:6	NS
Body mass index (kg/m^2)[a]	16.7–24.9 (20.8 ± 2.1)	18.5–24.7 (22.2 ± 1.9)	0.035
Alcohol ingestion (g/day)[b]	0–75 (25)	0–100 (0)	NS
ALT (nl. ≦36IU/*l*)[a]	9–78 (41 ± 20)	27–151 (75 ± 35)	0.0004
GGT (nl. ≦68IU/*l*)[a]	25–195 (68 ± 40)	20–238 (89 ± 59)	NS
Cholesterol (nl. ≦232 mg/*dl*)[a]	112–229 (156 ± 26.3)	107–193 (160 ± 44.7)	NS
Triglycerides (nl. ≦188mg/*dl*)[a]	34–128 (75 ± 21)	20–142 (92 ± 31)	0.042
Hemoglobin A1c (nl. ≦5.8%)[a]	3.1–5.7 (4.8 ± 0.66)	3.9–5.8 (4.8 ± 0.52)	NS
Histology (chronic hepatitis: cirrhosis)	9:13	4:17	NS
Serum HCV-RNA (log copies/*ml*)[b]	<2.0–5.9 (5.5)	<2.0–6.2 (5.7)	NS
Detection of HCV core protein (yes:no)	8:14	19:2	0.008

*ALT, alanine-aminotransferase; GGT, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; NS, not significant.
[a]Mean ± standard deviation.
[b]Median.

which has recently been shown to induce experimentally hepatic steatosis [Moriya et al., 1997; Barba et al., 1997; Moradpour et al., 1996], was selected. The association of the HCV core protein with the formation of fat droplets in the liver of patients with chronic hepatitis C was examined by evaluating both the histological findings and the presence of core protein at detectable levels in the liver tissue.

PATIENTS AND METHODS

Patients

Forty-three consecutive patients (33 males and 10 females, mean age ± S.D. = 65.7 ± 6.3 years) with chronic hepatitis C (13 chronic hepatitis and 30 cirrhosis) who underwent hepatectomy for hepatocellular carcinoma between July 1995 and September 1997 at the Second Department of Surgery, Tokyo University Hospital (Table I) were studied. All patients were positive for anti-HCV by the second-generation enzyme immunoassay (Dainabot, Tokyo, Japan), and none were positive for HBsAg (Auszyme II, Dainabot). Table I shows the clinical profile of the patients. Only one patient had a history of excessive alcohol consumption [exceeding 80 g per day; Sherlock and Dooley, 1997]. Careful history-taking excluded the use of medicines that may produce a fatty liver. The experimental protocol was approved by the Ethics Review Committee for Human Experimentation, and written informed consent was obtained from each patient.

Liver Samples and Evaluation

Liver tissue samples obtained at hepatectomy were processed for evaluation as follows. A liver tissue sample (approximately 10 mg), excised from a noncancerous part of the hepatectomized liver, was divided into two pieces. One piece was processed for protein analysis, and the other was fixed in 10% neutral buffered formalin and processed for hematoxylin-eosin staining. Microscopic analysis of the specimens was done blindly by two of the study group members. The degree of hepatic steatosis was graded as follows [Lefkowitch et al., 1993]: grade 0, absent; grade 1, mild, corresponding to steatosis in less than 1/3 of the hepatocytes; grade 2, moderate, with steatosis in 1/3 to 2/3 of the hepatocytes; and grade 3, marked, with steatosis in more than 2/3 of the hepatocytes.

Antibodies and Western Blotting

Monoclonal antibody was raised against the partially purified recombinant core protein expressed by baculovirus [Moriya et al., 1997]. For the analysis of the core protein, the liver tissue was homogenized for Western blotting in sample buffer (5% β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, and 1 mM EDTA). The samples were separated in 12.5% SDS/polyacrylamide gel and electro-transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) as described previously [Koike et al., 1995]. The filter was then reacted with anti-core monoclonal antibody, followed by anti-mouse IgG conjugated with horseradish peroxidase (Vector Labs, Inc., Burlingame, CA) and visualized by an ECL kit (Amersham Intl., Buckinghamshire, UK).

The specificity of the core protein detection by our Western blotting system has been confirmed using positive and negative controls [Moriya et al., 1997, 1998]. This system can detect as low as 0.5 ng of the core protein as titrated by fluorescent enzyme immunoassay [Yasui et al., 1998].

Serum HCV-RNA Levels

Serum samples were collected at the time of hepatectomy and stored at –80°C until use. Quantitation of HCV-RNA was performed using an Amplicor-HCV monitor kit (Nippon Roche, Tokyo, Japan). Its sensitivity is 1,000 copies/ml.

Statistical Analysis

Chi-square test with Yates' correction, Welch's *t*-test, or Mann-Whitney's *U* test was used where appropriate. Multiple logistic regression model analysis was used to determine the best predictive model for hepatic steato-

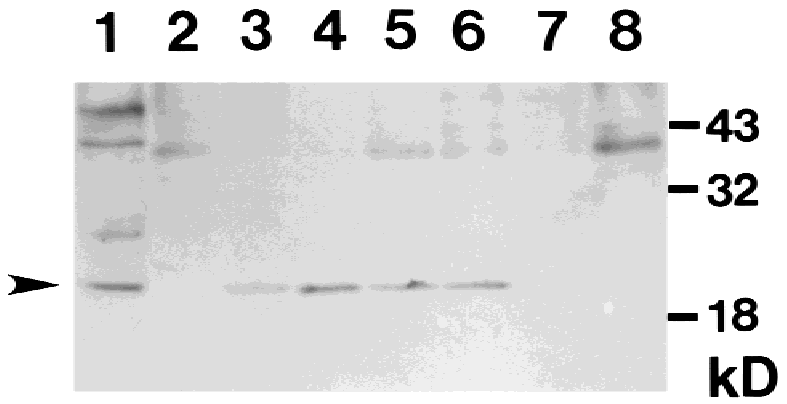

Fig. 1. Detection of the core protein in the liver of patients with chronic hepatitis C by Western blot analysis. Lane 1, positive control core protein expressed in the liver of a mouse transgenic for the HCV core gene; lane 2, liver sample from a patients with chronic hepatitis B as a negative control; lanes 3–8, liver samples from patients with chronic hepatitis C. Arrowhead indicates the position of the core protein. Numbers on the right side indicate the molecular weight standard in kilodaltons (kDa).

sis and to identify independent factors that correlated with it.

RESULTS

Intrahepatic Levels of the Core Protein and Hepatic Steatosis

Hepatic steatosis was found in 21 (49%) of the 43 patients (Table I). Hepatic steatosis was graded by microscopic examination as follows: grade 0 in 22 patients, grade 1 in 14, grade 2 in 6, and grade 3 in 1. Such distribution is more or less consistent with that reported previously [Scheuer et al., 1992; Bach et al. 1992; Lefkowitch et al. 1993; Czaja and Carpenter, 1993; Delladetsima et al., 1996].

Western blot analysis using anti-core mouse monoclonal antibody detected the HCV core protein of the expected size, 22 kDa, in 27 (63%) of the 43 samples. Representative cases are shown in Figure 1. Almost two thirds of those chronic hepatitis C patients with detectable hepatic core proteins had high levels of core protein in the liver (Fig. 1, lanes 3–6), while the remaining patients were also assumed to have the core protein in the liver but at lower levels. It is noteworthy that the core protein levels in most of the patients who were positive for core protein were comparable to those expressed in the liver of mice transgenic for the HCV core gene [Moriya et al., 1998]. The core protein was detected in 8 (36%) of the 22 patients without steatosis (grade 0), and 19 (90%) of the 21 with steatosis (grades 1–3) ($P = 0.008$, Table I). In terms of the core protein positivity, steatosis was observed in 19 (70%) of the 27 core protein-positive patients, while it was found in only 2 (13%) of the 16 core protein-negative patients. The steatosis in these two core protein-negative patients was grade 1 (mild). No significant correlation was observed between the intensity of the core protein band and the grade of steatosis.

Univariate analysis showed that in addition to the presence of the core protein at detectable levels ($P = 0.008$), the serum alanine aminotransferase (ALT) level, body mass index (BMI), and serum triglycerides level were also significant parameters correlated with the presence of steatosis ($P = 0.0004$, $P = 0.035$, and $P = 0.042$, respectively, Table I), whereas other parameters such as the histological diagnosis, alcohol ingestion or the serum total cholesterol level were not significant (Table I). Alcohol consumption in patients with steatosis was not significantly higher than in those without steatosis (mean, 28 vs. 26 g/day) in the present study.

Multivariate analysis using a logistic regression model showed that the following three were found to be independent parameters: the serum ALT level, BMI and core-protein positivity (Table II).

TABLE II. Independent Factors Associated With Hepatic Steatosis*

Factor	Odds ratio	95% CI	*P*
ALT value (IU/L)			
1: ≤65	1		
2: >65	26.62	2.13–331.94	0.013
Body mass index (kg/m^2)			
1: ≤22.0			
2: >22.0	17.28	1.23–242.89	0.0378
Detection of HCV core protein			
1: no			
2: yes	11.61	1.18–113.78	0.0384

*Data are by multiple logistic regression model. The maximum log likelihood of this model was −13.87.
CI, confidence interval.

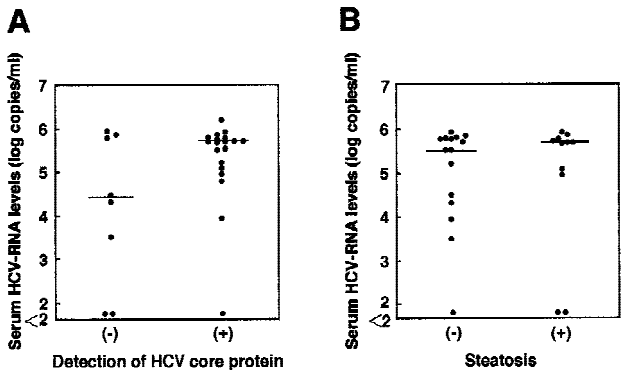

Fig. 2. Relationship between serum levels of HCV-RNA and the detection of intrahepatic HCV core protein (A), and the presence of hepatic steatosis (B). **A:** Serum HCV-RNA levels were slightly higher in patients with detectable HCV core protein but the difference was not significant ($P = 0.254$). **B:** There was no significant difference in serum HCV-RNA levels between patients with and without steatosis ($P = 0.640$). The horizontal bar in each column indicates the median.

Serum HCV-RNA Levels

The levels of HCV-RNA in the sera collected at the time of hepatectomy were slightly higher in patients with detectable intrahepatic core protein than in those without it (Fig. 2A), but the difference was not statistically significant ($P = 0.254$, Mann-Whitney U test). There was no significant difference in serum HCV-RNA levels between patients with and without steatosis ($P = 0.640$, Fig. 2B).

DISCUSSION

When compared with chronic hepatitis B or autoimmune chronic active hepatitis, chronic hepatitis C is characterized by several unique histologic features such as bile duct damage, lymphoid follicles, and steatosis. Among these features, steatosis is observed in 52 to 72% of liver tissues obtained from chronic hepatitis C patients [Scheuer et al., 1992; Bach et al., 1992; Lefkowitch et al., 1993; Czaja and Carpenter, 1993; Delladetsima et al., 1996]. The prevalence and degree of steatosis observed in the present study are compatible with those reported previously. It should be noted that steatosis occurs at a high rate in chronic hepatitis C patients, even those with cirrhosis; two thirds of our patients had histologically proven cirrhosis.

In the present study, a close relationship was demonstrated between the presence of steatosis and the detection of intrahepatic core protein, which is supposed to represent the HCV load in the liver. Detection of the core protein by Western blotting, which indicates a high level of expression of this protein in the liver, was an independent determinant of the presence of steatosis in the liver of patients with chronic hepatitis C. The results indicate that the presence of HCV itself in the liver is an important determinant for the development of steatosis.

A number of conditions have been known to be risk factors for hepatic steatosis, including alcohol excess, obesity, starvation, parenteral hyperalimentation, diabetes mellitus, pregnancy, and certain drugs [Sherlock and Dooley, 1997]. While univariate analysis showed that the serum ALT level, detection of the core protein, BMI and serum triglycerides level were significant determinants of the presence of steatosis, only the former three were found to be independent parameters by multivariate analysis. Alcohol consumption did not appear to be a significant parameter because in the present study only one patient had a history of excessive alcohol consumption. It was rather unexpected that the serum ALT level was an independent parameter that correlated with the presence of steatosis in both univariate and multivariate analysis. Aminotransferases, released from hepatocytes following their death, are parameters of hepatocyte damage and turnover. Steatosis in chronic hepatitis C hence also may be partly dependent on events that accompany cell death and regeneration, such as the production of free radicals [Farinati et al., 1995; Arthur et al., 1985].

The levels of serum HCV-RNA were slightly higher in patients with detectable intrahepatic core protein than in those without it, but the difference was not statistically significant. Previous reports have indicated an inconsistency in the relationship between the levels of HCV-RNA in serum and HCV-RNA or HCV protein in the liver. They demonstrated significant [Nakagawa et al., 1994; Lau et al., 1996], weak [Gonzalez-Peralta et al., 1995; Sakamoto et al., 1994], or no correlation [Ballardini et al., 1997] between the levels of HCV-RNA in the serum and HCV(-RNA) in the liver. This inconsistency may be attributable to heterogeneity in the intrahepatic distribution of HCV, "sampling error" in obtaining liver tissue samples, and the sensitivity of the methods used to detect intrahepatic HCV(-RNA). Our current findings on the relationship between the levels of serum and liver HCV are compatible with previous reports. On the other hand, in the present analysis, there was no significant difference in serum HCV-RNA levels between patients with and without steatosis, which negates any association between serum HCV-RNA level and hepatic steatosis. The topical intrahepatic concentration of the HCV, and not the serum concentration, is probably associated with the development of steatosis in the liver of patients with chronic hepatitis C.

The core protein of hepatitis C virus has been shown to induce hepatic steatosis in mice transgenic for the core gene of HCV [Moriya et al., 1997]. Also, in vitro studies have shown that cultured cells transfected with the core gene showed the accumulation of lipid droplets in the cytoplasm, on the surfaces of which the HCV core protein was localized [Barba et al., 1997; Moradpour et al., 1996]. These experimantal studies suggest an important role of the core protein in the development of fatty change of the liver. Our current finding, therefore, may indicate an essential role of the core protein in the pathogenesis of steatosis, although the core protein levels detected in our study may only represent the amount of HCV. The mechanism of HCV replication including the regulation of viral protein synthesis and

intracellular distribution of the proteins [Suzuki et al., 1996; Yasui et al., 1998] must be further studied to elucidate the precise role of the core protein in human HCV infection.

In conclusion, the results indicate a close relationship between the presence of steatosis and in situ level of the HCV. Since a recent experimental transgenic mouse study strongly connects the HCV core protein and steatosis with hepatocarcinogenesis [Moriya et al., 1998], further analysis of the core protein in human liver tissues may facilitate the elucidation of the mechanism of liver diseases in HCV infection.

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